

Dissertation for the Doctor of Philosophy Degree

Evaluation of Resveratrol and  
Tocotrienols as potential REDOX –  
active compounds for Cardioprotection

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## INTRODUCTION

Since cardiovascular disease contributes in a major way to the morbidity and mortality, it is becoming a strain on the economy of many countries worldwide. Although various factors have been identified as possible causes of different cardiac diseases such as heart failure and ischemic heart disease, there is a real need to elucidate their role for the better understanding of the cardiac disease pathology and formulation of strategies for developing newer therapeutic interventions. These include myocardial shunts, valvular disease, long-standing hypertension, intracardiac or intravascular shunts, cardiac arrhythmias and hyperthyroidism, as well as other causes. Perhaps the most common cause of heart failure due to systolic and diastolic dysfunction is ischemic heart disease.

### Ischemic Heart Disease

Ischemia is a stage when there is no blood flow in a cell; as blood is the only carrier of air or oxygen, that cell is undergoing a lot of stress due to lack of oxygen. When this kind of situation arises in the heart, that disease is known as Ischemia Heart Disease. Ischemic heart disease is also known as Coronary Artery Disease, because the two coronary arteries are the major supplier of the blood in the myocardium. This disease occur due to the deposition of cholesterol plaques on the wall of these blood vessels and make those vessels narrow and ultimately getting block by those fatty deposit. This process is called Atherosclerosis, which leads ischemia to the heart muscle and this can cause damage to the heart muscle. The complete blockage of the arteries leads to myocardial infraction (MI). According to the World Health Organization, the major cause of death in the world as a whole by the year 2020 will be acute coronary occlusion.

Myocardial ischemia occurs when there is an imbalance between requirements and the availability of blood and oxygen in the myocardial cells. Apart from atherosclerosis, there are numerous risk factors are involved for this Myocardial Ischemia and Hypoxia. Among them smoking, diabetes mellitus, Hypercholesterolaemia, severe hypotension as in shock, aortic stenosis, Genetic and hereditary factors may also be responsible for the disease. Severe anemia may be an additional factor.

Angina pectoris is the primary symptom of ischemic heart disease, caused by transient episodes of ischemia, which is the imbalance in the myocardial oxygen supply-demand relationship and increase in myocardial oxygen demand (determined by heart rate, ventricular wall tension and ventricular contractility), by a decrease in myocardial oxygen supply (determined by coronary blood flow).

### Cardiac arrhythmias

Acute myocardial infarction leads to a lethal condition called cardiac arrhythmias. Amongst Ventricular Tachycardia (VT) or and Ventricular Fibrillation (VF), VF is considered the major cause of premature death, called sudden cardiac death, both in North America as well as in U.K. VF may occur within a min or hour or months after the onset of angina pectoris. VF can occur in two separate phases, first reversible injury and then the irreversible injury which leads to the infarct zone.

The mechanism for cardiac arrhythmias is not very clear yet, there is considerable controversy going on among the researchers. Calcium overload, Sodium overload and oxidative stress due to free radical formation are the major regulator of fetal cardiac arrhythmias. Although evidence exists for a role for oxygen free radicals in the pathogenesis of reperfusion-induced arrhythmias some of them may be circumstantial, controversial or open to alternative interpretation. Recent studies showed the various intracellular mechanisms by the generation of free radical leads to the cardiac arrhythmias. Therefore now-a-days oxidative stress is considered as the major cause of cardiac arrhythmias.

### Cardiac Oxidative Stress

Although reperfusion of the ischemic myocardium during early stages is essential to prevent cardiac damage, reperfusion of the ischemic heart after a certain critical period has been reported to have deleterious effects due to the generation of reactive oxygen species (ROS). Ischemia reperfusion injury may also occur after the termination of an anginal attack, there is vasospasm, platelet aggregation or collateral blood flow perfusion. Since myocardial ischemia has been shown to serve as an initial signal for the development of acute and chronic heart failure at later stages, it is believed that oxidative stress plays a significant role in different types of cardiac diseases. This view is further substantiated upon observing the beneficial effects of antioxidants in hearts subjected to ischemia-reperfusion. This oxidative stress-induced

cellular damage has been estimated by measuring the levels of lipid peroxidation through different detection methods involving malondialdehyde (MDA) or thiobarbituric acid. Moreover, exposure of the heart or subcellular organelles to oxyradical generating systems has been reported to produce effects similar to those observed in hearts subjected to ischemia-reperfusion.

In ischemic-reperfused hearts, the increase in oxidative stress was observed to correlate well with cardiac dysfunction, a decrease in the antioxidant defense mechanisms and an increase in lipid peroxidation, leading to increased membrane permeability. An increase in the levels of MDA and decreased activities of superoxide dismutase (SOD) and catalase have been reported in hearts exposed to 30 min of ischemia. Similar increases in the oxidative stress level were observed in the ischemic-reperfused hearts with normal levels of antioxidant activities. Regional differences were observed in the glutathione peroxidase levels, which were normal in the left ventricle of the ischemic and reperfused heart, but were increased in the right ventricle. Hearts treated with SOD plus catalase showed a decrease in infarct size, an improvement in cardiac function and sarcoplasmic reticular regulatory function associated with  $\text{Ca}^{2+}$ /calmodulin protein kinase. The depressed SL  $\text{Ca}^{2+}$ -pump,  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchange and  $\text{Na}^{+}$ - $\text{K}^{+}$  ATPase activities in the ischemia-reperfused hearts were also prevented with a combination of SOD plus catalase.

### Redox Signaling

ROS are produced endogenously as a consequence of normal cell functions or derived from external sources. They pose a threat to cells living in an aerobic environment because they can result in DNA, protein and lipid damage. As mentioned previously, ROS also play an important role in the pathophysiology of many diseases, including ischemic heart disease. All cells contain a number of antioxidant defense mechanisms to minimize fluctuations in ROS, however, when ROS generation exceeds a cell's antioxidant capacity, the result is a condition known as oxidative stress. A host's survival then depends upon the ability of its cells and tissues to either adapt to or resist this stress. A number of stress response mechanisms have evolved to protect cells from oxidative insult and these mechanisms are rapidly activated. Some of these pathways are preferentially linked to enhanced survival, while others are associated with cell death. In ischemic heart disease, ROS, along with abnormal lipid metabolism and calcium homeostasis,

gives rise to the “death signal” resulting in apoptotic cell death that leads to an infarcted heart. The mammalian heart is also protected against ischemic injury by several lines of defense. The first line of defense consists of intracellular antioxidants such as superoxide dismutase, catalase and other protective enzyme systems. Recently, it has become apparent that the heart produces oxidative stress-inducible proteins in an attempt to counteract the invading ROS and that these proteins can also function as a defense system. I-R injury is likewise associated with the induction of a number of both pro- and anti- apoptotic genes and transcription factors.

#### Ischemic Preconditioning

Interestingly it has been found that patients with one or few episode of angina pectoris earlier could make the heart more tolerant from this deadly condition. After more than 15 years of extensive research work, researcher finally can explain this fact. The underline cause of this fact they termed as “Ischemic Preconditioning”. Ischemic preconditioning is the protective adaptive phenomenon produced by short periods of ischemic stress resulting in a marked, albeit temporary, resistance of the myocardium to a subsequent more prolonged period of that same insult. It was first described by Murry and colleagues in 1986 showing that preconditioning the dog heart with four brief periods of ischemia, each 5 min in duration, caused the heart to tolerate a subsequent 40-min ischemic insult with less fraction of the infraction than that realized in nonpreconditioned hearts. Since this report, many other researchers have demonstrated the profound protective effect that can be achieved with either a single short cycle of ischemia and reperfusion or a number of such cycles preceding a prolonged ischemic episode.

#### Antioxidants

Antioxidants are the molecules that scavenge the free radicals from the cells, by donating one electron. It also may work as terminator of the free radical generator by reducing the energy of the free radical or by interrupting an oxidizing chain reaction to minimise the damage cause by the free radicals.

There are several enzymes which normally released from the various tissues, including superoxide dismutase (SOD), catalase and glutathione peroxidase, that neutralise the effect of free radicals. Apart from the natural antioxidant produced from

the tissues there are some minerals like Mg, Zn, Se and Cu which act as an antioxidant by inducing the secretion of these enzymes that act as antioxidants. Mg, Zn and Cu acts as a building block (helps to form the enzymes) nutrients for SOD secretion and Se for glutathione peroxidase. In addition to enzymes, many vitamins and some other minerals act as potent antioxidants, such as various polyphenolic compounds like vitamine E, vitamine C, uric acid, beta-carotene, lutein, lycopene, vitamin B<sub>2</sub>, co enzyme Q<sub>10</sub> and cysteine (an amino acid). Herbs such as bilberry, turmeric, gapes seed, pine bark extracts, palm oil, rice bran oil, green tea and ginkgo bilova can also provide powerful antioxidant protection. Another example is NAC (N-acytilecystine), a cell permeable potent antioxidant.

### Resveratrol

The current popular propositions about the benefits of “moderate wine drinking” dates back through history and was first proposed by the Father of Medicine, Hippocrates of Kos in Greece. Epidemiological and experimental studies have revealed that mild-to-moderate drinking of wine, particularly red wine, attenuates the cardiovascular, cerebrovascular, and peripheral vascular risk. However, the experimental basis for such health benefits is not fully understood. The cardiovascular benefits of red wine became the hub of research activity after the observation of the ‘French paradox’ by Renaud and associates who, in 1992, found that there was a low mortality rate from ischemic heart disease among French people despite their high consumption of saturated fats and the prevalence of other risk factors such as smoking. This was attributed to so-called ‘Mediterranean diet’, which includes a large intake of wine. The cardioprotective effect of wine has been attributed to both components of wine; the alcoholic portion and more importantly, alcohol-free portion containing antioxidants. Wines are manufactured from grapes, which also contain a large variety of antioxidants including resveratrol, catechin, epicatechin and proanthocyanidins. Resveratrol (trans-3, 4', 5-trihydroxystilbene) is mainly found in the grape seeds and grape skin.

Recently, a number of studies have demonstrated that resveratrol given prior to ischemic arrest could protect the heart from ischemia and/or reperfusion injury. The role of nitric oxide (NO) is found to be one of the important mechanisms of pharmacological preconditioning by resveratrol. Another study has indicated that

adenosine receptors have an important function in the resveratrol preconditioning. In another study, HO-1 has been reported to play an important role in resveratrol preconditioning. Apart from this, the same study showed that resveratrol may protect the heart from ischemia-reperfusion injury by angiogenesis. Resveratrol attenuates various soluble intercellular cytokines like ICAM, VCAM and e-Selectin through improvement in the endothelium function which reduces the infarct size. Apart from the REDOX signaling mechanism, resveratrol also protects the heart as a potent antioxidant by scavenging free radicals and inhibiting lipid peroxidation both in vitro and in vivo. Thus, resveratrol inhibits apoptotic cell death as well as release and/or generation of inflammatory mediators. Some of the mechanisms for the cardioprotective effects of resveratrol are depicted.

#### Tocotrienols

Vitamin E was discovered in 1922 at Berkeley University in California. It was first found to play a vital role in the fertility of rats. The first component identified was  $\alpha$ -tocopherol. It was named as such from the Greek *tokos* (*offspring*) and *pheros* (*to bear*). It is the most abundant form of vitamin E found in blood and body tissue. Vitamin E actually refers to a family of eight antioxidants; four tocopherol isomers ( $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -) and four tocotrienol isomers ( $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -). Tocotrienols and tocopherols differ from each other in their aliphatic tail. Tocopherols have a saturated side chain where as tocotrienols have unsaturated side chains. The isomers of tocotrienol and tocopherols differ in the methyl substitution on their chromayl ring. Vitamin E tocotrienols are abundant in cereal grains including soy beans, oats, rice, bran as well as in palm oil. Recent studies have shown many health benefits of tocotrienols including anticancer and tumor-suppressive activities as well as inhibition of lipid peroxidation in biological membranes. Dietary tocotrienols are derived from plant sources, mainly palm oil from palm fruits. The tocotrienol-rich fraction (TRF) from palm oil is composed primarily of 26%  $\alpha$ -tocopherol, 26%  $\alpha$ -tocotrienol, 36%  $\gamma$ -tocotrienol, and 12%  $\delta$ -tocotrienol.



## AIMS OF THIS STUDY

Based on the reports from other laboratories and previous work done by our own research group, the overall objective of this study was to describe that with the therapeutic dose resveratrol and tocotrienols can reduce the ischemia/reperfusion injury, and serve as a cardioprotective agent. In this approach we utilized several strategies such as the investigation of free radicals scavenging properties of resveratrol and tocotrienols in order to protect cardiomyocytes from the oxidative stress, determination of optimal dose and time-points for the cardioprotective effect of resveratrol and tocotrienols, investigation of some pro-apoptotic protein degradation, in addition to this investigation of the pharmacological preconditioning effect of resveratrol and tocotrienols and the underlying intracellular signaling pathways; with special emphasis on the redox signaling mechanisms.

The specific aims were:

1. To further evaluate resveratrol and tocotrienols as cardioprotective compounds by first determining the therapeutic dose of these compounds for and then carefully examining their role in preconditioning by following analysis :
  - Cardiac function analysis
  - Infarct Size determination
  - Apoptosis
  - Neucrosis
2. To examine the survival pathways of intracellular signaling by resveratrol and tocotrienols by studying potential molecular targets of death vs. survival signaling pathways including PI3Kinase, Akt, MAPKinase, p38 MAPKinase, cSrc, cJUN, cJNK, PKC and their downstream Transcription Factor gene including NFkB, Bcl-2, Bad, CREB etc.
3. By using specific inhibitors of each protein target, to evaluate the survival pathway and reconfirming the cardioprotective mechanism of resveratrol and tocotrienols.
4. To determine potential redox regulation of resveratrol and tocotrienols by examining their in-vivo antioxidant effects using either ESR or MDA formation.

## MATERIALS AND METHODS

### 1. Materials

Resveratrol (trans-3, 4', 5-trihydroxystilbene), a natural phytoalexin, was obtained from Sigma Chemical Co. (St. Louis, MO, USA). A highly specific blocker of the adenosine A<sub>3</sub> receptor MRS 1191 (3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate) and PD 098,059 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one), a MEK inhibitor and p38MAPK blocker, SB202190 (4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole) were also purchased from the same company. LY 294002 (2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride), a PI-3-kinase inhibitor and MSK-1 blocker, H-89 (N-[2-(p-Bromocinnamyl-amino)ethyl]-5-isoquinolinesulfonamide dihydrochloride) were purchased from Calbiochem Corp. (San Diego, CA, USA). All chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise mentioned. Tocotrienol Rich Factor (TRF) was supplied by the Malaysia Palm Oil Board. The drugs were dissolved in DMSO, and the aliquots were kept at 4°C. Control experiments used the vehicle (0.01% DMSO) only.

### 2. Animals

All animals used in this study received humane care in compliance with the principles of the laboratory animal care formulated by the National Society for Medical Research and Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (Publication Number NIH 85-23, revised 1985). Sprague Dawley male rats weighing between 250-300 gm were fed *ad libitum* regular rat chow with free access to water until the start of the experimental procedure. The rats were randomly assigned to one of the following groups: pre-perfused the isolated hearts for 15 min with KHB i) containing 0.1% DMSO as a control group; ii) KHB containing 10 µM resveratrol; iii) 10 µM resveratrol + 1 µM MRS 1191; iv) 10 µM resveratrol + 3 µM LY 294002; v) 10 µM resveratrol + 20 µM PD098059 vi) 10 µM resveratrol + 20 µM 098059 + 3 µM LY 294002; vii) 10 µM resveratrol + 10 µM SB 202190; or viii) 10 µM resveratrol + 1 µM H-89. All hearts were then subjected to 30 min ischemia followed by 2 h reperfusion. Control experiments were performed with vehicle

(DMSO) only, MRS 1191 only, LY 294002 only or PD 098059 only or SB 202190 only or H 89 only.

For TRF study the groups are: i) control (vehicle); ii) 0.035 % TRF; iii) 5  $\mu$ M PPI or iv) 0.035 % TRF + 5  $\mu$ M PPI. TRF was dissolved in ethanol.

### 3. Isolated working heart preparation

Rats were anesthetized with sodium pentobarbital (80 mg/kg, i.p.), (Abbott Laboratories, North Chicago, IL, USA) and anticoagulant with heparin sodium (500 IU/kg, i.v.) (Elkins-Sinn Inc., Cherry Hill, NJ, USA). After ensuring sufficient depth of anesthesia thoracotomy was performed, hearts were perfused in the retrograde Langendorff mode at 37 °C at a constant perfusion pressure of 100 cm of water (10 kPa) for a 5 min washout period. The perfusion buffer used in this study consisted of a modified Krebs-Henseleit bicarbonate buffer (KHB). The Langendorff preparation was switched to the working mode following the washout period as previously described. At the end of 10 min, after the attainment of steady state cardiac function, baseline functional parameters were recorded. The circuit was then switched back to the retrograde mode and hearts were perfused either KHB with vehicle or adenosine A<sub>3</sub> receptor antagonist or the other inhibitors (Control), Resveratrol at a concentration of 10  $\mu$ M or a combination of resveratrol and adenosine A<sub>3</sub> receptors antagonist or the inhibitors for a duration of 15 min. In case of TRF study, after the steady state of the heart in the apparatus the heart were perfused with vehicle (ethanol) or PPI (control), TRF at a concentration 0.035% or a combination of TRF and PPI. This was followed by a 5-min washout with KHB buffer, and then the hearts were subjected to global ischemia for 30 min and then 2 h of reperfusion. The first 10 min of reperfusion was in the retrograde mode to allow for post ischemic stabilization and there after, in the anti-grade working mode to allow for assessment of functional parameters, which were recorded at 10-, 30-, 60- and 120- min reperfusion.

### 4. Cardiac function assessment

Aortic pressure was measured using a Gould P23XL pressure transducer (Gould Instrument Systems Inc., Valley View, OH, USA) connected to a side arm of the aortic cannula, the signal was amplified using a Gould 6600 series signal conditioner and monitored on a CORDAT II real-time data acquisition and analysis system (Triton

Technologies, San Diego, CA, USA). Heart Rate (HR), Left Ventricular developed pressure (LVDP) (defined as the difference of the maximum systolic and diastolic aortic pressures), and the first derivative of developed pressure (dP/dT) were all derived or calculated from the continuously obtained pressure signal. Aortic flow (AF) was measured using a calibrated flow-meter (Gilmont Instrument Inc., Barrington, IL, USA) and coronary flow (CF) was measured by timed collection of the coronary effluent dripping from the heart.

#### 5. Infarct size estimation

At the end of reperfusion, a 10 % (w/v) solution of triphenyl tetrazolium in phosphate buffer was infused into aortic cannula for about 10 min at 37 °C. The hearts were excised and stored at -70 °C. Sections (0.8 mm) of frozen heart were fixed in 2% Para formaldehyde, placed between two cover slips and digitally imaged using a Microtek ScanMaker 600z. To quantitate the areas of interest in pixels, a NIH image 5.1 (a public-domain software package) were used. The infarct size was quantified and expressed in pixels.

#### 6. TUNEL Assay for assessment of Apoptotic Cell Death

Immunohistochemical detection of apoptotic cells was carried out using TUNEL assay by using APOP TAG® kit (Oncor, Gaithersburg, MD). After 2 hr of reperfusion, the heart tissues were immediately put in 10% formalin for 24-48 hrs then the tissues were washed with water and put it in 70 % ethanol for dehydration and finally fixed in an automatic tissue-fixing machine. The tissues were carefully embedded in the molten paraffin in metallic blocks, covered with flexible plastic moulds and kept under freezing plates to allow the paraffin to solidify. The metallic containers were removed and tissues became embedded in paraffin on the plastic moulds. Prior to analyzing tissues for apoptosis, tissue sections were deparaffinized with xylene and washed in succession with different concentrations of ethanol (absolute, 95%, 70%). Then tissues were incubated again with mouse monoclonal antibody recognizing cardiac myosin heavy chain to specifically recognize apoptotic cardiomyocytes. The fluorescence staining was viewed with a confocal laser microscope. The number of apoptotic cells was counted and expressed as a percent of total myocyte population.

## 7. Western blot Analysis

Left ventricles from the hearts were homogenized in a buffer containing 25 mM Tris-HCl, 25 mM NaCl, 1 mM orthovanadate, 10 mM NaF, 10 mM pyrophosphate, 10 mM okadaic acid, 0.5 mM EDTA and 1 mM PMSF. High-molecular-weight markers (Bio-Rad, Hercules, CA, USA) and 50  $\mu$ g total membrane proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred onto 0.45- $\mu$ m polyvinylidene difluoride (PVDF) membrane. PVDF membrane was blocked overnight at 4°C in Tris-buffered saline (TBS) containing 5% skim milk and probed with phospho-Akt, Akt, phospho-ERK 1/2, ERK 1/2, phospho-p38MAPK, p38MAPK, phospho-MAPKAPK2, MAPKAPK2, phospho-MSK1, MSK1, CREB, and p-CREB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for overnight at 4°C. Primary antibodies were diluted in TBS-T according to the manufacturer's instructions. Horseradish peroxidase-labeled anti-mouse or anti-rabbit (according to the manufacturer's instructions) IgG (Bio-Rad, CA, USA) was also diluted according to the manufacturer's instructions in TBS-T and used as a secondary antibody. Band intensities of the Western blot were quantified using a CCD camera imaging densitometer (Bio-Rad GS 800). The linearity of the Western blot procedure used for the quantification in subsequent blotting experiments 50  $\mu$ g membrane protein was used because it is in the linear range. The resulting blots were digitized, subjected to densitometric scanning using a standard NIH image program, and normalized against loading control.

## 8. Proteasome activity

Proteasome activity was determined in cell lysate. Frozen cardiac tissue was homogenized in HEPES buffer (137 mM NaCl, 4.6 mM KCl, 1.1 mM  $\text{KH}_2\text{PO}_4$ , 0.6 mM  $\text{MgSO}_4$ , 1 mM EDTA, 1 mM DTT, and 0.01% digitonin) without protease inhibitors at 4°C and then centrifuged at 10,000 g to obtain the soluble fraction. Cell supernatant (50  $\mu$ g of protein) was incubated in 50 mmol/l Tris-HCl buffer, pH 7.8, containing 20 mM KCl, 0.5 mM  $\text{MgCl}_2$ , and 1 mM DTT for 1 h with 75  $\mu$ M succinyl-LLVY-methylcoumarin (Biomol Research Laboratory, Plymouth Meeting, PA). Hydrolysis was stopped by addition of ice-cold ethanol and dilution with 0.125 mol/l sodium borate, pH 9.0, and fluorescence products were monitored at 380-nm excitation and 440-nm emission. The reaction was carried out in the absence and presence of the

proteasome inhibitor lactacystin (5  $\mu$ M; Biomol Research Laboratory) to differentiate between nonproteasome- and proteasome-mediated peptide hydrolysis and with or without 0.0625–0.125 mmol/l ATP (with or without lactacystin) to differentiate between 20S and 26S proteasome, respectively. Results are expressed as percentage of control, because storage of tissue samples, even at  $-80^{\circ}\text{C}$ , can result in interassay variation. Care was taken to avoid freeze thawing of tissue samples more than once and to match experimental samples with preischemic controls that had been stored for similar amounts of time.

#### 9. Statistical analysis

The values for myocardial functional parameters, total and infarct volumes and infarct sizes and cardiomyocyte apoptosis are all expressed as the mean  $\pm$  standard error of mean (SEM). Analysis of variance test followed by Bonferoni's correction was first carried out to test for any differences between the mean values of all groups. If differences between established, the values of the treated groups were compared with those of the control group by a modified t-test. The results were considered significant if  $p < 0.05$ .

## RESULTS AND DISCUSSION

### 1. Effects of Resveratrol on Myocardial Function

There were no differences in baseline function amongst all the thirteen groups. In general, there were no significant differences between resveratrol vs. control, PD98059, SB202190, LY 294002, MRS 1191 and H-89 as well as vs. Resveratrol + PD98059, Resveratrol + SB202190, Resveratrol + LY 294002, Resveratrol + MRS 1191 or Resveratrol + H-89 vs. Resveratrol on heart rate and coronary flow. As was expected, upon reperfusion, the absolute values of all functional parameters were decreased in all the groups as compared with the respective baseline values. Resveratrol group displayed significant recovery of post ischemic myocardial function. Aortic flow was markedly higher in the resveratrol group from R-30 onwards at the all rest three points; R-30 ( $66.1 \pm 3.62$  ml/min vs.  $36.02 \pm 12.7$  ml/min), R-60 ( $43.5 \pm 5.34$  ml/min vs.  $19.24 \pm 6.48$  ml/min) and R-120 ( $14.9 \pm 2.36$  ml/min vs.  $4.28 \pm 1.43$  ml/min). The cardio protective effects of resveratrol were evidenced by significant differences in the LVDP from R-30 onwards at the all rest three points, the difference was especially apparent at R-30 ( $121.7 \pm 2.64$  mm Hg vs.  $103.57 \pm 7.1$  mm Hg), R-60 ( $110.27 \pm 1.2$  mmHg vs.  $88.02 \pm 9.57$  mmHg) and at R-120 ( $87.8 \pm 1.74$  mm Hg vs.  $52.5 \pm 4.56$  mm Hg). For LVdp/dt, resveratrol mediated increased recovery was apparent at R-60 ( $2843 \pm 79.48$  mmHg/sec vs.  $1880.5 \pm 403.3$  mm Hg/sec) and at R-120 ( $1391.8 \pm 104.7$  mm Hg/sec vs.  $899.83 \pm 86.75$  mmHg/sec). With the use of PD098059, SB202190 and LY 294002, resveratrol partially lost its cardio-protective effect, but with the combination of PD + LY, MRS 1191 and H-89, resveratrol significantly lost its cardio protective effects, which were evidenced by significant differences in the post ischemic period of LVDP from R-30 onwards at all of the three time points. PD 098,059 or SB 202190 or LY 294002 did not decrease the LVDP at R-30 or at R-60 level but with H-89 and Res + PD + LY the decrease was prominent both at R-30 ( $105.5 \pm 5.78$  mm Hg and  $103 \pm 2.8$  mm Hg, respectively, vs.  $121.7 \pm 2.64$  mm Hg) and R-60 ( $83.9 \pm 4.75$  mm Hg and  $84 \pm 2.3$  mm Hg, respectively, vs.  $110.27 \pm 1.2$  mm Hg) and with MRS 1191 the decrease is significant at R-60 onwards, at R-60 ( $97.9 \pm 4.2$  mm Hg vs.  $110.27 \pm 1.2$  mm Hg). At R-120 the decrease is significant with all the inhibitors, PD 098059, SB 202190, LY 294002, MRS 1191, the combination of PD and LY and H-89, [R-120 ( $61.35 \pm 4.05$  mmHg,  $68.62 \pm 3.59$  mmHg,  $70 \pm 12$  mm Hg,  $75.7 \pm 3.7$  mm Hg,  $59 \pm$

4.7 mm Hg and  $60.63 \pm 6.27$  mmHg, respectively, vs.  $87.8 \pm 1.74$  mmHg)]. The same effect of PD 098,059, SB 202190, LY 294002, MRS 1191, PD + LY and H-89 on resveratrol preconditioning also reflects from the significant decrease of LVdp/dt at R-60 ( $2114.7 \pm 119.29$  mmHg/sec,  $1760.5 \pm 158.44$  mmHg/sec,  $1908.67 \pm 249.55$  mmHg/sec,  $2,077 \pm 286.4$  mmHg/sec,  $1716 \pm 92$  mmHg/sec and  $1633.17 \pm 225.59$  mmHg/sec, respectively, vs.  $2843 \pm 79.48$  mmHg/sec) and at R-120 ( $877.7 \pm 187.27$  mmHg/sec,  $866.7 \pm 96.61$  mmHg/sec,  $932.33 \pm 207.45$  mmHg/sec,  $980.16 \pm 62.5$  mmHg/sec,  $707 \pm 71$  mmHg/sec and  $862.3 \pm 126.32$  mmHg/sec, respectively, vs.  $1425.2 \pm 109.72$  mmHg/sec). But with PD + LY and H-89 the significant decrease also observed at R-30 ( $2623 \pm 27$  mmHg/sec and  $2566.5 \pm 143.17$  mmHg/sec, respectively, vs.  $3012.7 \pm 64.02$  mmHg/sec) apart from the other time points. This was also confirmed from the Aortic flow value; which is markedly lower at R-30 onwards at all the rest two time points with all the inhibitors.

## 2. Effects of TRF on ventricular function

There were no differences in baseline function among the six groups. In general, there were no significant differences between TRF and control in heart rate and coronary flow. As expected, on reperfusion, the absolute values of all functional parameters were decreased in all groups compared with the respective baseline values. TRF displayed significant recovery of postischemic myocardial function. The cardioprotective effects of TRF were demonstrated by significant differences in the left ventricular dP/dt from 30 min of reperfusion onward, the difference is especially apparent at 60 and 120 of reperfusion and also in left ventricular developed pressure at 120 min of reperfusion. AF was markedly higher in the TRF group from 30 min of perfusion onward. This is also confirmed from the AF value, which is markedly lower throughout the reperfusion period. Similar to TRF, PPI also improved postischemic ventricular recovery.

## 3. Effects of Resveratrol on Myocardial Infarct size

Infarct size (percent of infarct vs. total area at risk) was noticeably reduced in resveratrol group as compared to the control ( $18.17 \pm 2.08\%$  vs.  $34.7 \pm 2.74\%$ ). This infarct zone was increased significantly when resveratrol were used along with PD 98,059, SB 202190, LY 294002 and MRS 1191 ( $29.8 \pm 1.98\%$ ,  $30.4 \pm 2.44\%$ ,  $24.9 \pm$



2.3 % and  $26.33 \pm 2.45$  %, respectively, vs.  $18.17 \pm 2.08$ %). When resveratrol was used along with the combination of (PD + LY) and H-89 the infarct zone was further increased compared to the other inhibitors ( $30.05 \pm 4.9$  % and  $33.6 \pm 2.62$  %).

#### 4. Effects of TRF on myocardial infarct size

Infarct size was significantly higher in the hearts subjected to 30 min of ischemia and 2 h of reperfusion than in the hearts that were not subjected to the ischemia-reperfusion protocol (almost at the baseline level, data not shown). The values were noticeably reduced in TRF and PPI groups compared with the group subjected to ischemia-reperfusion:  $25.1 \pm 2.45$  and  $25.6 \pm 2.33$ %, respectively, vs.  $33.43 \pm 2.44$ %. The infarct size was further reduced when a combination of TRF and PPI was used ( $20.5 \pm 2.2$ % vs.  $33.43 \pm 2.44$ %).

#### 5. Effects of Resveratrol on Cardiomyocyte Apoptosis

The percent of apoptotic cardiomyocytes was significantly reduced in resveratrol group as compared to the control ( $3.7 \pm 1.2$ % vs.  $22.7 \pm 1.5$ %). This apoptotic cell death was increased significantly when resveratrol was used along with MRS 1191. Thus, the apoptosis was significantly higher in the resveratrol + MRS1191 group as compared to the resveratrol group ( $20.9 \pm 1.7$ % vs.  $3.7 \pm 1.2$ %). Inhibition of PI-3-kinase with LY 294002 or MEK with PD 098059 or p38 MAPK with SB 202190 also increased the number of apoptotic cardiomyocytes to ( $17.4 \pm 1.2$ %,  $18.9 \pm 1.4$  % and  $11.8 \pm 1.6$ %, respectively) compare to resveratrol group. The cardiomyocyte apoptosis was further increased when the combination of PD 098059 and LY 294002 and H-89 were used with resveratrol ( $24.3 \pm 1.1$  % and  $23.6 \pm 2.1$  %).

#### 6. Effects of TRF on cardiomyocyte apoptosis

Ischemia-reperfusion caused the cells to undergo apoptosis, as expected. The percentage of apoptotic cardiomyocytes was significantly reduced in the TRF and PPI groups compared with the control group:  $5.7 \pm 1.3$  and  $6.6 \pm 2.2$ %, respectively, vs.  $22.0 \pm 1.7$ %. As observed for the infarct size, the apoptotic cardiomyocytes were also further reduced when a combination of TRF and PPI was used:  $4.8 \pm 0.8$ % vs.  $22.0 \pm 1.7$ %.

7. Effects of TRF on ventricular arrhythmias

The total incidence of VF (sustained and nonsustained) was significantly reduced with TRF and PPI from its control value of 90% to 30% and 40%, respectively, indicating antiarrhythmic effects of tocotrienol and PPI.

8. Effects of Resveratrol on the phosphorylation of Akt, p38MAPK, MAPKAP kinase2, MSK-1 and CREB

Resveratrol significantly enhanced the phosphorylation of Akt and CREB. Phosphorylation of Akt was increased by 10-12 fold and CREB by 6-7 folds. The resveratrol-mediated induction of Akt and its subsequent phosphorylation was reduced significantly by MRS 1191 and LY 294002, but not with PD 098059. In contrast, any one of the three blockers inhibited the phosphorylation of CREB. LY 294002 and PD 098059 partially, but MRS 1191 and LY294002 plus PD 098059 almost completely abolished resveratrol mediated CREB phosphorylation.

Resveratrol significantly enhanced the phosphorylation of MAP kinases. Phosphorylation of ERK1/2, p38 MAP kinase, and MAPKAP kinase 2 was increased significantly as compared to control. Resveratrol-mediated increased phosphorylation of ERK1/2 was reduced by PD98059, but not with SB202190, increased phosphorylation of p38MAP kinase and MAPKAP kinase was reduced by SB202190, but not with PD98059.

The phosphorylation pattern of MSK-1 and CREB is shown in Figure 6. Resveratrol increased the phosphorylation of both MSK-1 and CREB. Increased phosphorylation of MSK-1 and CREB was reduced significantly by either PD98059 or SB202190. Resveratrol-mediated increased phosphorylation of MSK-1 and CREB was almost abolished by H-89.

9. Effects of TRF on c-Src expression and phosphorylation

Because previous studies indicated negative regulation of c-Src with vitamin E, we determined whether TRF could reduce the ischemia-reperfusion-induced upregulation of c-Src. The results show that ischemia-reperfusion significantly increased the induction and phosphorylation of c-Src activities. TRF minimally, but significantly, affected the ischemia-reperfusion-mediated increase in c-Src induction.

TRF-mediated reduction of c-Src phosphorylation was highly significant. PPI completely abolished c-Src expression (data not shown).

10. Effects of TRF on ischemic and postischemic proteasome activities

20S and 26S proteasome activities were significantly ( $P < 0.05$ ) depressed by 45 and 46%, respectively, after ischemia. Preischemic treatment of hearts with TRF not only prevented this decrease, but it actually appeared to activate both proteasomes to levels significantly ( $P < 0.05$ ) higher than the ischemic values. After 120 min of reperfusion, 20S proteasome activity recovered to a level not different from baseline, and TRF had no effect. However, 26S proteasome activity was still significantly ( $P < 0.05$ ) depressed by 58%, which was prevented in the TRF-treated hearts. Treatment of hearts with PPI had no protective effects on postischemic proteasome activities (data not shown).

## CONCLUDING REMARKS

We have initiated a study aimed at identifying the sub-cellular mechanism of action of resveratrol and tocotrienols, derived from palm oil, in the pathogenesis of ischemic heart disease and furthermore to reveal the redox signaling pathways for the cardioprotection of these two natural polyphenols. In order to accomplish these goals, we have utilized several strategies in order to investigate the cardioprotective role of resveratrol and tocotrienols derived from palm oil.

Our major findings were the following:

- Resveratrol preconditioning is mediated by adenosine A<sub>3</sub> receptors that trigger CREB phosphorylation via both PI3-kinase-Akt and via MEK-CREB pathways. Resveratrol-mediated phosphorylation of Akt and CREB was blocked by MRS-1191, which also abolished cardioprotective abilities of resveratrol, indicating a crucial role of adenosine A<sub>3</sub> receptor for resveratrol preconditioning. That LY294002 completely inhibited Akt phosphorylation but partially blocked the phosphorylation of CREB, resulting in partial inhibition of resveratrol's ability to precondition the heart, suggests that PI3-kinase-Akt-CREB signaling pathway is at least partially responsible for the cardioprotection achieved by resveratrol. Partial blockage of CREB phosphorylation and resveratrol-mediated cardioprotection by PD098059 indicates negative role of PI3-kinase/Akt signaling in CREB activation. This receives further support from the finding that LY294002 and PD098059 together abolished the phosphorylation of CREB simultaneously inhibiting resveratrol-mediated cardioprotection. The results indicate that resveratrol preconditions the hearts through adenosine A<sub>3</sub> receptor signaling that triggers the phosphorylation of CREB through both Akt-dependent and -independent pathways, leading to cardioprotection.
- The results of the present study showed for the first time that resveratrol triggers a preconditioning-like survival signaling by activating MAP kinase signaling pathway. Thus, resveratrol activates both ERK1/2 and p38MAPK both of which contributes towards the phosphorylation of MSK-1. There appears to be two downstream targets for p38MAPK, MSK-1 and MAPKAP kinase 2. MSK-1 in turn activates CREB, which was previously shown to transmit survival signal by activating Bcl 2.

- Ischemia-reperfusion caused ventricular dysfunction, electrical rhythm disturbances, and increased myocardial infarct size. PPI or TRF could reverse the ischemia-reperfusion-mediated cardiac dysfunction. Ischemia-reperfusion also upregulated c-Src expression and phosphorylation. Although TRF only minimally affected c-Src expression, it significantly inhibited the phosphorylation of c-Src. Ischemia-reperfusion reduced 20S and 26S proteasome activities, an effect prevented by TRF pretreatment. PPI exerted a cardioprotective effect that is not mediated by the proteasome but, rather, through direct inhibition of c-Src. The results of this study support a role for c-Src in postischemic cardiac injury and dysfunction and demonstrate direct cardioprotective effects of TRF. The cardioprotective properties of TRF appear to be due to inhibition of c-Src activation and proteasome stabilization.

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